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Nerve Sprouting in Muscle Is Induced and Guided by Processes Extended by Schwann Cells

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Summary

Partial denervation or paralysis with botulinum toxin, manipulations that induce sprouting of nerve terminals in muscle, also induced terminal Schwann cells to extend processes. These processes were associated with every nerve sprout and in some cases were longer than the sprouts that appeared to be growing along them. Following partial denervation, more than 70% of the nerve sprouts that grew to innervate nearby denervated endplates were associated with Schwann cell processes that had extended from the denervated endplates, i.e., in the direction opposite to nerve growth. Implantation of Schwann cells into an innervated muscle induced sprouting upon contact of an axon or nerve terminal by Schwann cell processes. These observations show that Schwann cells induce and guide axonal sprouting in muscle.

Introduction

A remarkable demonstration of synaptic plasticity is the ability of neurons in the CNS and PNS to sprout new processes and form additional synapses in response to nearby denervated targets (Tsukahara, 1981). Sprouting of muscle innervation has been studied with particular intensity (Brown et al., 1981a; Wernig and Herrera, 1986). Destruction of a portion of a muscle's innervation ("partial denervation") results in sprouting of the remaining undamaged axons and nerve terminals, followed by the innervation of denervated muscle fibers (cf. Thompson and Jansen, 1977).

A number of studies have sought to identify the stimuli for sprouting in muscle and the means by which sprouts manage to locate denervated endplates, the preferred site for synapse formation. Two types of observations suggest that the sprouting stimulus originates, in part, from lack of electrical/contractile activity in denervated muscle fibers. First, paralysis in the absence of denervation is able to induce sprouting (Duchen and Strich, 1968; Brown and Ironton, 1977; Holland and Brown, 1980). Second, sprouting induced by paralysis (Brown et al., 1980) or partial denervation (Brown and Holland, 1979) can be prevented by using extrinsic electrical stimulation. Paralysis and denervation are both known to result in up-regulation of cell surface and basal lamina components that promote axon growth (Sanes et al., 1986), and some of these components could serve to guide axonal sprouts to endplates. In addition, a number of exogenously administered trophic factors, including insulin-like growth factor-1, ciliary neurotrophic factor, and basic fibroblast growth factor, have been reported to induce sprouting (Caroni and Grandes, 1990; Gurney et al., 1992), although the source and role of these factors in situ is not yet completely clear.

Recent experiments have implicated Schwann cells in axon guidance during muscle reinnervation. These experiments employed a monoclonal antibody (MAb) that recognizes an epitope concentrated in the postsynaptic apparatus at the neuromuscular junction (Astrow et al., 1992). This postsynaptic epitope disappears following muscle denervation (Astrow et al., 1992) but appears in Schwann cells (Astrow et al., 1994), including those at the nerve terminal. This antibody was used as a Schwann cell marker to demonstrate that regenerating axons follow processes extended by Schwann cells in response to denervation (Son and Thompson, 1995 [this issue of *Neuron*]). Such guidance leads to growth of axons beyond endplates and polyneuronal innervation. Moreover, the rate of axonal regeneration from the cut end of a nerve is set by the rate at which the Schwann cells guiding these axons extend.

In view of the involvement of Schwann cells in axonal growth during regeneration, we investigated whether Schwann cells play a role in nerve sprouting. We examined the growth of Schwann cell and neuronal processes in partially denervated adult muscle, the response of nerve terminals and Schwann cells to paralysis by botulinum toxin, and the ability of Schwann cell processes growing in an otherwise normal muscle to induce nerve terminal and axonal sprouting. The results show that Schwann cells play a primary role in initiating sprouting and in guiding the growth of the resulting sprouts.

Results

Partial Denervation Causes Terminal Schwann Cells to Elaborate Processes

Partial denervation was performed using the soleus muscle of adult rats of the AO strain. In about 50% of the muscles of this strain, a portion of the soleus motor pool reaches the muscle by following a second "aberrant" pathway (Thompson and Jansen, 1977). The peripheral location of the two nerve branches to the muscle provides an easy method for partial denervation: resection of the usual nerve to soleus leaves intact the second, aberrant nerve that usually contains 2–10 motor neurons. Because the fibers innervated by the two nerves are intermixed, partial denervation leads to extensive sprouting of the remaining motor neurons; these neurons can expand to innervate up to five times their normal adult complement of fibers (Thompson and Jansen, 1977).

We confirmed the report of Mehta et al. (1993) that Schwann cells in partially denervated muscles elaborate processes. We double labeled muscles in whole mount with an antibody to the 200 kDa neurofilament protein and MAb 4E2. Denervated endplates in these muscles were identified by their lack of neurofilament staining and the reduction in postsynaptic immunoreactivity for 4E2. In 3

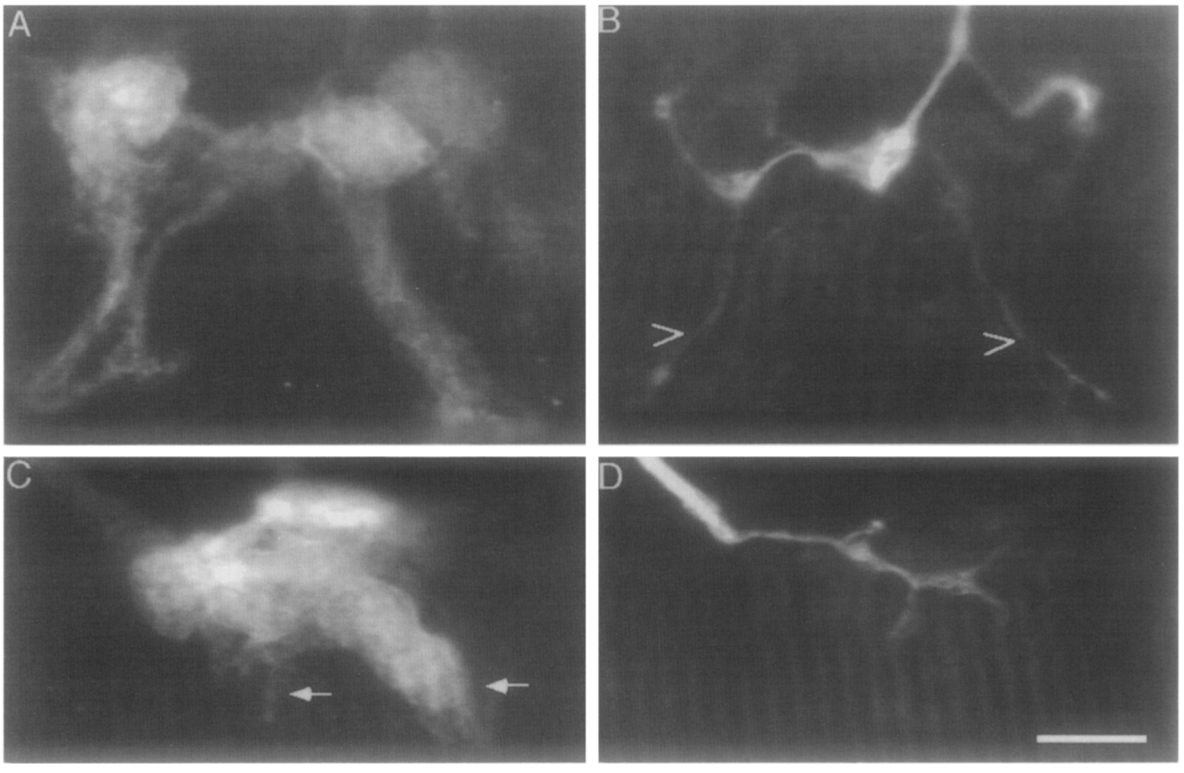

Figure 1. Partial Denervation Leads to Sprouting of Nerve Terminals and Elaboration of Schwann Cell Processes

Soleus muscle, partially denervated 3 days earlier, was double labeled with an antibody to S-100, a Schwann cell marker (A and C), and anti-neurofilament antibody (B and D). The junction in (A) and (B) has two prominent nerve sprouts (labeled "V" in [B]), and anti-S-100 labeling (A) shows that each sprout is associated with a terminal Schwann cell process. Extension of terminal Schwann cell processes was found in several instances (C, arrows) in the absence of obvious sprouting of the nerve terminal (D). Bar, 10 μm.

muscles examined 3 days after partial denervation, 99% of the denervated endplates (n = 719) had MAb 4E2–labeled Schwann cell processes extending more than 20 μm from the outlines of the endplate (average = 34.7 μm). Most of these endplates had several Schwann cell processes (an average of 7.8). In about 50 cases in these 3 muscles, MAb 4E2–immunoreactive processes had extended to reach adjacent innervated endplates (see below).

Innervated endplates in the partially denervated muscles were identified by the presence of a neurofilament-labeled nerve terminal and postsynaptic immunoreactivity for MAb 4E2. No processes immunoreactive for MAb 4E2 originated from these Schwann cells. However, MAb 4E2 marks Schwann cells that have lost contact with axons (Astrow et al., 1994). To examine the possibility that Schwann cells at these endplates had extended processes not labeled by MAb 4E2, we employed an antibody to S-100, a general marker for Schwann cells. This antibody revealed that Schwann cell processes were present at innervated endplates (Figures 1A and 1C). However, process growth by these Schwann cells was less vigorous than that of Schwann cells at denervated endplates. In 3 muscles examined 3 days after partial denervation, only 47% of 128 innervated endplates had Schwann cell processes that appeared to leave the endplate zone, and these processes tended to be shorter in length (average = 28.2 μm) and smaller in number per endplate (average = 3.0). The differences in sprout length and number at denervated versus innervated endplates were statistically significant (two-tailed t test, $p < .01$).

Nerve Terminal Sprouts Are Associated with Processes Extended by Terminal Schwann Cells

At 3 days following partial denervation, nerve sprouts had formed at 41% of the remaining innervated endplates (n = 128). Each of these sprouts was associated with an anti-S-100-labeled Schwann cell process(es) (Figures 1A and 1B). In several cases the Schwann cell processes were longer than the growing axons with which they were associated (data not shown), but in no case was the axon longer than the Schwann cell process. At a few innervated endplates, nerve sprouts were present on only a portion of the Schwann cell processes extended from these endplates. Moreover, about 6% of the 128 innervated endplates had elongated Schwann cell processes but showed no signs of terminal sprouting (Figures 1C and 1D).

Muscles double labeled with MAb 4E2 and anti-neurofilament 3 days after partial denervation confirmed the existence of terminal sprouts arising from some innervated endplates, but MAb 4E2 labeled no Schwann cell processes associated with these sprouts (with the exception of endplates to be discussed below). On the other hand,

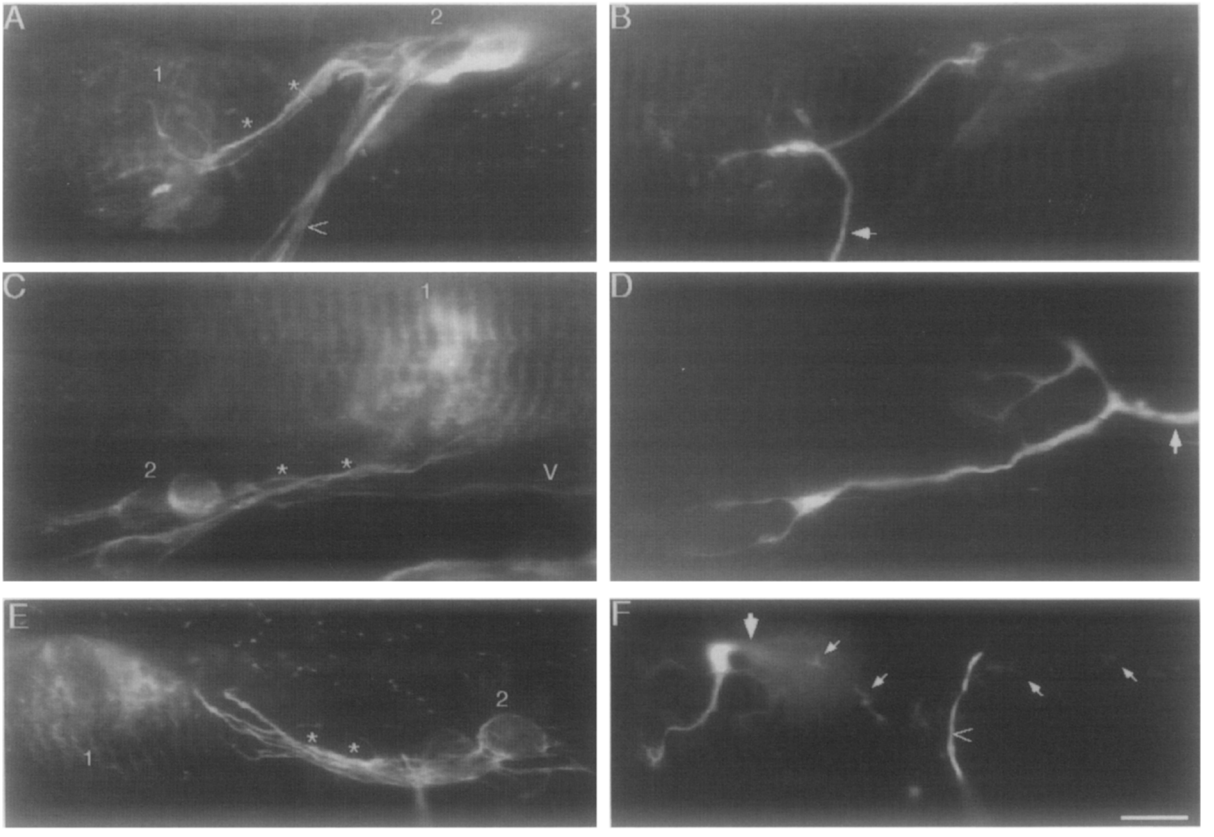

Figure 2. Terminal Sprouts Grow to Denervated Endplates by Following Processes Extended by Schwann Cells at Denervated Endplates

Muscle fibers 3 days after partial denervation were double labeled with MAb 4E2 (A, C, and E) and with anti-neurofilament antibody (B, D, and F). The top two pairs of panels (A and B, C and D) illustrate two cases in which a neurofilament-labeled nerve sprout has grown from an innervated junction (labeled "1") to a denervated junction (labeled "2") by following a MAb 4E2–labeled Schwann cell process (asterisks). The innervated junction was identified by the preterminal axon entering it (arrows), the presence of postsynaptic labeling with MAb 4E2, and the absence of labeling of the terminal Schwann cells by MAb 4E2. The denervated endplate was identified by the absence of postsynaptic labeling with MAb 4E2, the presence of MAb 4E2–labeled Schwann cell bodies, and a MAb 4E2–labeled Schwann cell tube devoid of any axon (labeled "V"). The bottom pair of panels illustrates a case in which Schwann cell processes from a denervated endplate have approached an innervated endplate that has not yet begun to sprout. MAb 4E2–labeled Schwann cell processes (asterisks) arising from a denervated endplate (labeled "2" in [E]) have extended to an area near the innervated junction (labeled "1"), but the nerve terminal of junction 1 lacks sprouts (F). The large arrow in (F) indicates the preterminal axon for endplate 1, which arises from deeper within the muscle and is mostly out of focus. The small arrows in (F) indicate nonspecific labeling of a blood vessel. The "V" in (F) marks an axon not involved in the innervation of endplate 1 or 2. Bar, 10 μm.

muscles labeled with anti-neurofilament and anti-S-100 antibodies show that every terminal sprout was associated with a Schwann cell process(es). We therefore conclude that MAb 4E2 marks a subset of the Schwann cell processes in partially denervated muscle: those processes extended by Schwann cells at denervated endplates.

Neuronal Sprouts Arrive at Denervated Endplates by Growth along Schwann Cell Processes

Even as early as 3 days after partial denervation, we found many instances in which axonal sprouts extended from an innervated endplate had approached or reached a denervated endplate (Figures 2B and 2D). Because MAb 4E2 selectively labels the Schwann cell processes extended from denervated endplates, we could determine the origin of the Schwann cell processes associated with these sprouts. The surprising finding was that most of these axonal sprouts were associated with Schwann cell processes extended from the denervated endplates.

In 3 muscles labeled with MAb 4E2 and anti-neurofilament antibody 3 days after partial denervation, we observed 61 pairs of endplates (1 innervated, 1 denervated) that had become interconnected by a sprout from a nerve terminal. In 72% of these cases (44/61 pairs), the axonal sprout was associated with a MAb 4E2–labeled Schwann cell process(es) (Figures 2A and 2B, 2C and 2D). Each of these 44 cases suggests that the Schwann cell process(es) grew from denervated endplates, contacted innervated endplates, induced the formation of a terminal sprout, and then guided that terminal sprout back to the denervated endplate. Of course, static images like these do not prove such a temporal sequence of events. However, a suggestion of such a sequence was found in observations of occasional endplates. In these cases, MAb 4E2–labeled

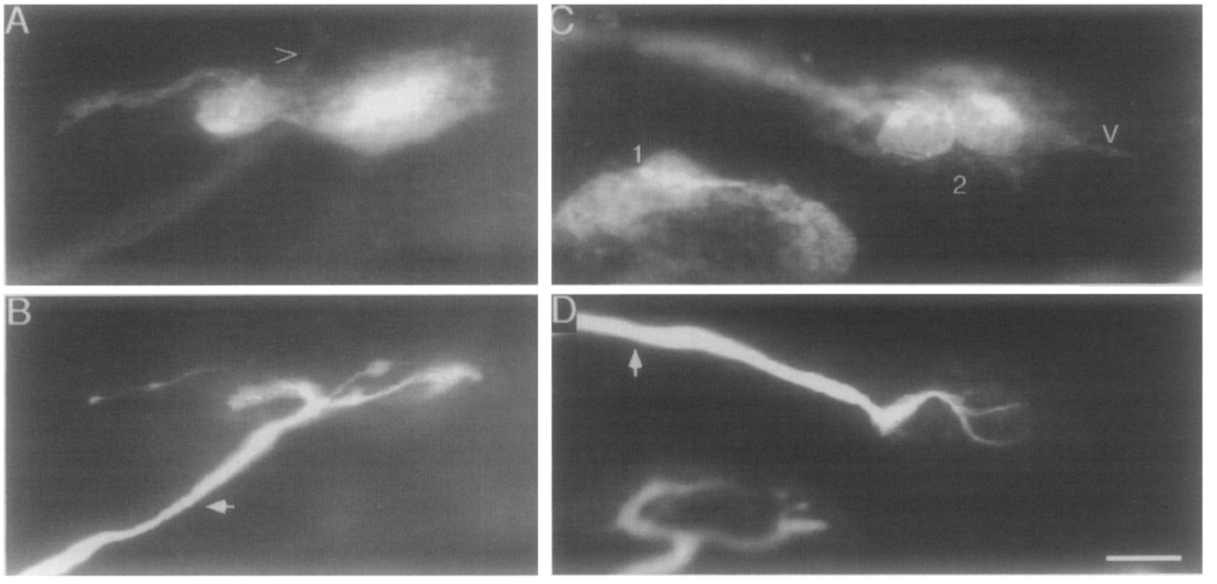

Figure 3. Botulinum Toxin Poisoning of Soleus Muscle Induces both Schwann Cell and Nerve Terminal Sprouting

Pairs of images obtained from muscles double labeled with anti-S-100 (A and C) and anti-neurofilament (B and D) antibodies. In (B), a nerve terminal sprout has extended from an endplate, and this sprout is associated with a Schwann cell process (A). In addition, some endplates possess Schwann cell processes (labeled "V" in [A] and [C]) that are not accompanied by nerve sprouts. Arrows in (B) and (D) mark the preterminal axon. (C) and (D) show 1 endplate (labeled "1") that exhibits no Schwann cell or neuronal sprouting. Bar, 10 μm.

Schwann cell processes extended from a denervated endplate had approached an innervated endplate, but no nerve terminal sprouts had yet formed (Figures 2E and 2F). These cases suggest a precedence for the formation of Schwann cell processes in terminal sprouting.

In the remaining 17 pairs of interconnected endplates, the neuronal sprouts were associated with either Schwann cell processes extended from the innervated endplate (10 pairs) or a mixture of processes extended from both denervated and innervated endplates (7 pairs). These conclusions were based on the presence or absence of MAb 4E2–labeled Schwann cell processes. Thus, for these pairs of endplates, it appears that the Schwann cells and axons had extended together from the innervated endplate for at least some distance.

Paralysis with Botulinum Toxin Causes both Nerve Terminal and Schwann Cell Sprouting

Application of botulinum toxin to the soleus muscle of 20- to 29-day-old rats resulted in nerve terminal sprouting as assayed by anti-neurofilament labeling of muscle whole mounts (Figure 3B). In these muscles we saw no evidence of Schwann cell labeling with MAb 4E2, probably because the Schwann cells were still in contact with their axons. However, anti-S-100 labeling showed that Schwann cell processes were associated with every terminal sprout (Figures 3A and 3B). In some cases we saw Schwann cell processes that were longer than the nerve terminal sprouts with which they were associated (data not shown). In a few cases we saw Schwann cell processes that had formed without nerve sprouts (Figure 3). The process formation following botulinum toxin–induced paralysis was less extensive than that following denervation. At 3 days after toxin treatment, only 38% of the endplates (n = 245) had Schwann cell processes extending more than 15 μm from the endplate, and these processes were fewer in number (most commonly 1 per endplate) and shorter (usually less than 30 μm) than the Schwann cells present at denervated endplates 3 days following partial denervation. This suggests that denervation may present additional stimuli for sprouting beyond those created by lack of muscle activity, a suggestion made previously in the literature (Brown et al., 1981a).

Schwann Cell Processes Can Induce Sprouting of Axons and Axon Terminals

Transplantation of a piece of nerve from which all axons have degenerated onto the soleus muscle results in a growth of Schwann cell processes and a migration of Schwann cells from the end of the nerve over the surface of the muscle (Son and Thompson, 1995). We wondered whether these Schwann cell processes would induce sprouting of nerve terminals and/or preterminal axons if they grew into the endplate zone. To examine this question, we transplanted a branch of the superficial fibular nerve to an area near the endplate zone of the soleus. The peroneal nerve was resected in the popliteal fossa 4–5 days before this transplantation in order to remove all axons. After allowing for Schwann cell sprouting and growth, we examined the muscles to determine how far Schwann cell processes had extended from the transplant and the status of axons and terminals in the endplate zone, by double labeling the muscles with MAb 4E2 and anti-neurofilament antibody.

We examined several transplants in which MAb 4E2–immunoreactive Schwann cell processes had approached

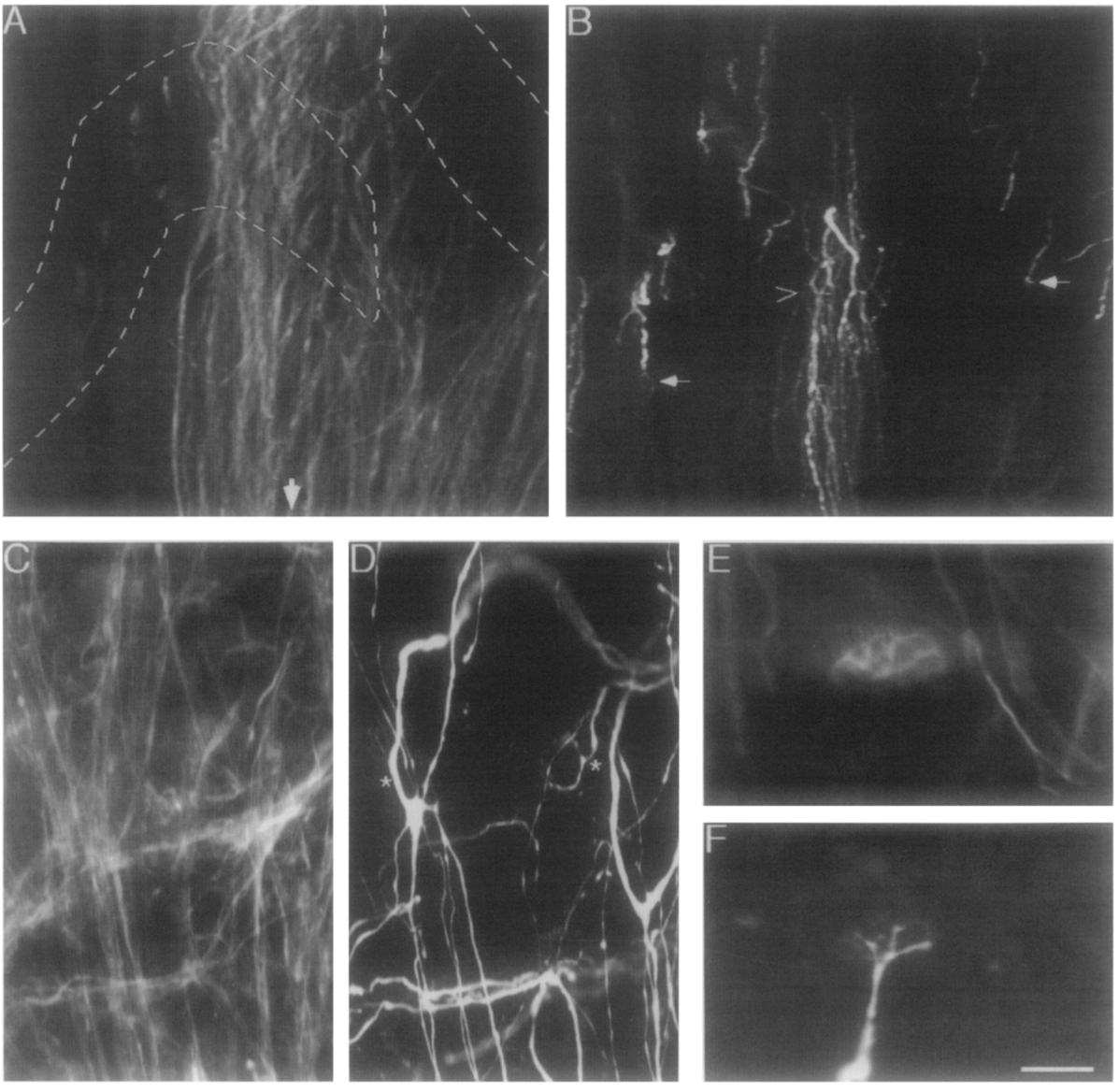

Figure 4. Schwann Cell Processes Induce Sprouting of Terminals and Axons upon Entering the Endplate Zone

(A) A nerve previously resected so that it contained no axons was transplanted to the surface of the soleus muscle. Schwann cell processes extending from the transplant were labeled with MAb 4E2 and have crossed the endplate band of the host muscle. This endplate band, which has a discontinuity, is outlined with dashed lines. The large arrow indicates the direction from which the processes have grown.

(B) Labeling of the axons and terminals in the same field as (A) with anti-neurofilament antibody. Axons have sprouted (labeled "V") onto the Schwann cell processes. Nerve terminals outside the growth zone of the Schwann cells have failed to sprout (two are indicated with small arrows).

(C and D) Higher magnification view of an area of (A) and (B), respectively. Two preterminal axons in (D) are marked with an asterisk at a point near their endplates. The one to the left has extensive terminal sprouting; the one to the right has two preterminal sprouts.

(E and F) Higher magnification view of an area of (A) and (B), respectively. Here a neuromuscular junction is surrounded by Schwann cell processes labeled with MAb 4E2 (E), but the terminal has not sprouted. The orientation of (E) and (F) is changed so that the muscle fibers are running horizontally, instead of vertically as in (A)–(D). Bar, 100 μm (A and B), 40 μm (C and D), 20 μm (E and F).

but had failed to reach the endplate zone of the muscle. In all of these cases (12 muscles with nerve transplants growing for 9–20 days), no sprouting was observed at the neuromuscular junctions (data not shown) other than that observed at an occasional junction in control muscles. This suggests that, if Schwann cells are able to induce nerve sprouting by release of diffusible agents, the action of these agents requires closer proximity and/or a longer time of exposure than those allowed in these experiments.

On the other hand, in 5 muscles the transplant had been placed nearer the endplates, and MAb 4E2–labeled Schwann cell processes had grown to reach some endplates (Figure 4A). In all of these muscles, nodal and terminal axon sprouts grew from the endplates that were con-

tacted by the Schwann cell processes. The sprouted axons grew from the endplate along the Schwann cell processes toward the transplanted nerve stump (Figure 4B).

In every case of sprouting observed in these experiments, the Schwann cell processes appeared to have contacted endplates or axons (Figures 4C and 4D), although in many of these cases, the Schwann cell growth was too dense to discern the relationship of individual nerves to Schwann cell processes. In many cases the processes had either grown over or passed near neuromuscular junctions, and these junctions failed to sprout (Figure 4E and 4F). Even in muscles in which Schwann cell processes had grown into the endplate and induced nerve sprouting, the sprouting was confined to the narrow zone of Schwann cell contact. These observations suggest the sprout-inducing action of the Schwann cells is contact mediated.

Discussion

The results presented here suggest a central role for Schwann cells in nerve sprouting. The majority of nerve sprouts connecting endplates in partially denervated muscle are apparently generated during the following sequence of events (Figure 5). Upon partial denervation, the Schwann cells at denervated endplates sprout processes, some of which contact intact terminals on adjacent muscle fibers. Such contact induces formation of terminal sprouts that grow along the Schwann cell process(es) to the adjacent denervated endplates, where synapses are formed. Sometime during growth of the sprouts, the remaining Schwann cell processes at denervated junctions are withdrawn.

The data presented in this paper are static images taken during sprouting. Though our data show a constant association between nerve sprouts and Schwann cell processes, this relationship has been apparent in previous studies of sprouting (Duchen, 1971; Wernig et al., 1984). Such an association does not show that Schwann cells lead, rather than merely accompany or follow, the extending axons. However, we believe our results provide compelling evidence that Schwann cells do in fact act as leaders. First, Schwann cells form processes in the absence of axons (Reynolds and Woolf, 1992; Astrow et al., 1994; Mehta et al., 1993; Son and Thompson, 1995) and thus are clearly capable of growing in advance of axons. In fact, Schwann cells extending in the absence of axonal sprouts were observed at several endplates after botulinum toxin poisoning. Second, MAb 4E2 provides a useful tool for studying sprouting, as it labels only those processes extended from Schwann cells at denervated endplates. Use of this antibody shows that the majority of endplate-to-endplate sprouting following partial denervation occurs in association with processes extended from Schwann cells at denervated endplates. Thus, at these endplates the direction of Schwann cell growth is opposite to axon extension. This suggests the sequence of events presented in Figure 5. Third, in a separate study, Son and Thompson (1995) have shown that terminal Schwann cell processes established before the beginning of reinnervation serve as pathways for regenerating axons. However, most compelling are the

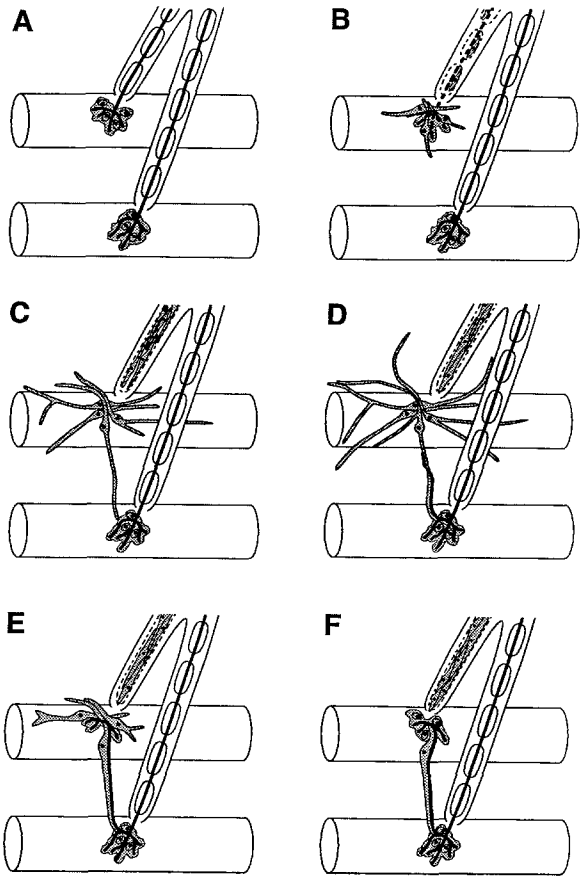

Figure 5. Hypothesized Role of Schwann Cells in the Most Common Form of Nerve Terminal Sprouting Following Partial Denervation

(A) Two endplates in the normal muscle; the nerve terminals are covered by terminal Schwann cells or their processes.

(B) Partial denervation interrupts the axon innervating the upper muscle fiber, resulting in degeneration of the nerve terminal and axon. In response to this denervation, the terminal Schwann cells on the denervated muscle fiber begin to grow processes.

(C) One of the terminal Schwann cell processes from the denervated endplate grows to reach the nerve terminal at the endplate of an adjoining muscle fiber.

(D) A sprout is induced from the nerve terminal and grows along the Schwann cell process toward the denervated endplate.

(E) The nerve sprout arrives at the denervated endplate, which it proceeds to reinnervate. The Schwann cell processes (except for the one associated with the sprout) are being withdrawn.

(F) Reinnervation of the denervated endplate is completed, and the Schwann cells again resume their coverage of the terminal branches.

results of experiments in which "axonless" nerves were transplanted onto the surface of an innervated muscle. Schwann cell processes growing from the transplanted nerve induced sprouting from the muscle's own nerve supply upon contact with the nerve terminals and axons in the endplate zone. Thus, Schwann cell processes do induce nerve sprouting. Together, these results argue that Schwann cells play a causal and not just a reactive role in nerve sprouting.

What Controls Schwann Cell Sprouting?

If Schwann cells induce and guide nerve sprouts, then what controls the sprouting of Schwann cells? An obvious

explanation is one commonly advanced for sprouting of neurons: the inactivity resulting from denervation or paralysis promotes nerve growth by changing the muscle's release of trophic factors or its expression of cell surface/basal lamina adhesion molecules (cf. Brown et al., 1981a; Sanes et al., 1986). Recent studies suggest that activity functions in a more direct communication between terminals and their Schwann cells: stimulation of motor axons produces calcium waves in terminal Schwann cells at frog neuromuscular junctions (Reist and Smith, 1992; Jahromi et al., 1992). This apparently results from transmitter release from the nerve terminal and acetylcholine or ATP binding to muscarinic receptors or adenosine receptors, respectively, on Schwann cells. Recently, Georgiou et al. (1994) have shown that blockade of neurotransmission in frog muscle leads to a rapid up-regulation in the expression of glial fibrillary acidic protein (GFAP) in terminal Schwann cells. GFAP is an intermediate filament protein critical for process formation in astrocytes (Weinstein et al., 1991). Our observation that botulinum toxin produces extension of processes by Schwann cells is consistent with such a neurotransmitter-regulated control of process extension exerted through regulation of GFAP.

However, certain results suggest that reduction in neurotransmitter release from nerve terminals is not the entire explanation for the elaboration of processes by Schwann cells. First, as shown above, Schwann cells extend processes even at some innervated endplates in partially denervated muscle, where neurotransmission is presumably unaltered. Second, Brown and his colleagues have shown that extrinsic stimulation of a botulinum toxin–poisoned muscle (Brown et al., 1980) or a partially denervated muscle (Brown and Holland, 1979) is able to suppress nerve terminal sprouting. In these cases, activity appears to act via the postsynaptic muscle fiber rather than through the release of neurotransmitter. Third, growth of Schwann cell processes (and up-regulation of GFAP) occurs in the myelinating Schwann cells of the nerve (Jessen and Mirsky, 1991), and this presumably cannot be effected via neurotransmitter release. Lastly, as shown above, the formation of Schwann cell processes is more robust from denervated endplates than from endplates that have been paralyzed by botulinum toxin. If the paralysis was complete, as we believe it to have been 3 days after toxin application, then some extra stimulus for Schwann cell sprouting must have been provided by denervation.

An obvious candidate for an additional stimulus for Schwann cell sprouting is loss of nerve contact. Nerve contact has potent effects on Schwann cells (Jessen and Mirsky, 1991). Upon loss of nerve contact, Schwann cells might be stimulated to grow beyond the growth that would occur from muscle paralysis alone. However, though this would explain why processes are extended by Schwann cells of the nerve following nerve lesions (Payer, 1979), it would not explain the sprouting of terminal Schwann cells present at innervated endplates. This sprouting apparently requires some kind of diffusible signal from denervated fibers. One possibility is that the Schwann cells at denervated endplates emit a signal(s) that induces sprouting of other Schwann cells. Upon loss of axonal contact, Schwann cells are known to up-regulate the synthesis of several trophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and ciliary neurotrophic factor (Heumann et al., 1987; Meyer et al., 1992; Sendtner et al., 1992; Friedman et al., 1992).

Is the Growth of Schwann Cell Processes Random?

In its simplest version, our hypothesis for Schwann cells and nerve sprouting proposes a random growth of Schwann cell processes. However, in many cases the Schwann cell processes do not appear to be randomly oriented. For example, in cases in which we believe Schwann cells had grown to and induced sprouting from an innervated endplate, few processes were seen to emerge in directions other than those oriented toward the innervated endplate (see Figures 2A, 2C, and 2E). If processes oriented in other directions were present, they tended to be shorter. One possible explanation for this orientation is that tropic molecules attract Schwann cell processes. Examples of such tropic attractions guiding nerve growth in muscle are present in the literature (Kuffler, 1989; Diaz and Pecot-Dechavassine, 1980), and these could be acting through Schwann cells (Chen and Ko, 1994). Recently, Van Mier and Lichtman (1994) have provided evidence of a tropic molecule released by regenerating muscle fibers.

However, a random outgrowth is appealing in its simplicity. Random outgrowth could be followed by a non-random stabilization of some processes, e.g., a retention of those processes that contact an axon, an endplate, some molecule in the junctional basal lamina, or another Schwann cell and a retraction of those processes that fail to do so. Some data favor such an explanation. The processes extended by Schwann cells in totally denervated muscle initially appear random, except for an increased tendency to grow along the long axis of the muscle fibers rather than across the fibers (Reynolds and Woolf, 1992; Son and Thompson, unpublished data). Schwann cell processes may grow and retract fairly rapidly. Our estimate for Schwann cell extension from the cut end of a nerve is about 0.2 mm per day (Son and Thompson, 1995). If this rate applies to terminal Schwann cells, the short distances between many endplates in our study (on the order of 100 μm) could be bridged in less than a day. If withdrawal of Schwann cell processes is equivalently rapid, and if stabilization of one set of processes signals others to withdraw, then a non-random orientation could probably be achieved over the course of 3 days. Resolution of this issue will require examination of events at earlier times or the application of repeated in vivo imaging (Magrassi et al., 1987) using a vital marker for Schwann cells.

Schwann Cells and the Control of Muscle Innervation

Our experiments suggest an additional possibility for how Schwann cells might influence muscle innervation. Since Schwann cells appear to play a major role in promoting nerve growth, they might also play a role in regression of nerve processes. Retraction of Schwann cells and their processes could cause axons to regress. If so, Schwann

cells, in addition to promoting extension of nerve processes and synapse formation, might have a role in the remodeling of nerve processes and synapse elimination. This possibility might be investigated by examining the retraction of Schwann cell processes and the axons associated with them during recovery from paralysis or upon reinnervation (Son and Thompson, 1995).

Experimental Procedures

Animals

Two strains of rats at various ages were employed. Both sexes were used. Partial denervation was performed on 100–150 g rats of the AO strain. Botulinum experiments were performed on Wistar rats 20–29 days old. Nerve transplantation experiments involved the use of adult Wistar rats (100–200 g). All operations were performed aseptically under ether anesthesia.

Partial Denervation

Partial denervation was performed by resecting a 1 mm piece of the lateral gastrocnemius-soleus nerve in the popliteal fossa at the head of the gastrocnemius.

Botulinum Toxin–Induced Paralysis

As Brown et al. (1981b) have shown that botulinum toxin–induced sprouting in rat muscles is much greater in younger (16–31 days) than in older adult animals, we employed rats 20–29 days old. Botulinum toxin A (4–6 ng; #203674, Calbiochem, La Jolla, CA) was dissolved in 10 μl of phosphate-buffered saline containing 0.2% gelatin. The endplate region of the soleus muscle in one hindlimb was exposed under ether anesthesia, and 10 μl of toxin-containing solution was applied; control littermates received an equivalent volume of solution lacking toxin. The solution was left in place for 5 min; the excess fluid was then removed, and the wound was closed with sutures. Following this application, the toe spreading reflex in the treated hindlimb was absent within a day. The animals were examined 2–6 days following treatment. At the time of the acute experiment, the rat was anesthetized with ether, and the muscle nerve was stimulated in situ while contractions or their absence was noted in the muscle.

Implantation of Resected Nerves

A 3–4 mm piece of the common peroneal nerve was resected in the popliteal fossa proximal to where the nerve crosses the lateral surface of the gastrocnemius. After 4 days, a branch of the superficial fibular nerve (itself a branch of the common peroneal) was dissected and cut, and its proximal tip was reflected to the surface of the soleus muscle. Attempts were made to dissect a piece of nerve long enough to allow its distal tip to lie close to the endplate zone of the soleus muscle.

Antibodies and Immunocytochemistry

Procedures for immunocytochemistry were those reported in a companion paper (Son and Thompson, 1995).

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